Comparative analysis and generation of a robust HIV-1 DNA quantification assay

Running title: Robust method for HIV-1 DNA Quantification

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Summary

Currently there is no standard HIV-1 DNA quantification assay for use in clinical trials and a comparison of HIV-1 assays has not been performed. Here, we evaluate two HIV-1 DNA quantification assays for their ability to accurately quantify HIV-1 DNA load in patient samples. Additionally, we have compared a number of commonly used HIV-1 latency cell for their use as HIV-1 quantification standards. We have shown that the two assays perform comparably and correlate strongly, however, one assay performs significantly better and this assay was chosen for improvement. We have also shown that J-Lat are the most suitable HIV-1 quantification standard owing to the stability of the integrated viral genome. We have redesigned primers based on analyses of the HIV-1 database to update and improve the HIV-1 quantification assay.

ABSTRACT HIV-1 infection cannot be cured due to the presence of the latent reservoir (LR). Novel cure or treatment strategies, such as “shock and kill” or therapeutic vaccination, aim to reduce or eradicate the LR. Cure strategies utilise robust DNA quantification assays to measure the change in the LR in low copy scenarios. No standard assay exists, which impedes the reliable comparison of results from different therapy and vaccine trials and HIV-1 total DNA quantification methods have not been previously compared. The HIV-1 LTR (long terminal repeat) has been shown to be the best target for DNA quantification. We have analysed two HIV-1 quantification assays, both able to differentiate between the variant HIV-1 DNA forms via the use of pre-amplification and primers targeting LTR. We identify a strong correlation (r=0.9759, P<0.0001) between assays and which is conserved in low copy samples (r=0.8220, P<0.0001) indicating that these assays may be used interchangeably. The RvS assay performed significantly (P=0.0021) better than the CV assay when quantifying HIV-1 total DNA in patient CD4+ T lymphocytes. Sequence analysis demonstrated that viral diversity can limit DNA quantification, however *in silico* analysis of the primers indicated that within the target region nucleotide miss-matches appear infrequently. Further *in silico* analysis using up to-date sequence information led to the improvement of primers and enabled us to establish a more broadly specific assay with significantly higher HIV-1 DNA quantification capacity in patient samples (p=0.0116, n=17).

Key Words: HIV-1 DNA quantification, latent reservoir, HIV-1 cure, HIV-1 therapeutic vaccines

Abbreviations: RvS, Rene van der Sluis assay; CV, Claire Vandergeeten assay; CRx, Christine Rouzioux

1. Introduction

The development of active antiretroviral therapy (ART) has been a major breakthrough in the treatment of human immunodeficiency virus type 1 (HIV-1) infection, effectively preventing the progression to acquired immunodeficiency syndrome (AIDS) (Brechtl et al., 2001). Despite this, ART cannot completely eradicate the virus due to the presence of a replication competent latent reservoir (LR) in different cell populations including long-lived resting CD4+ T cells that harbour pro-viral DNA integrated into the genome (Chun et al., 1997a; Finzi et al., 1997). Such infected cells can produce replication competent HIV-1, supporting rapid viral rebound following ART interruption (Davey et al., 1999; Joos et al., 2008; Rothenberger et al., 2015). Research is therefore focused on the development of novel approaches to reduce or eliminate the LR, with the aim of developing a functional cure for HIV-1 infection.

Therapeutic vaccination, administered during ART mediated virus suppression aims to stimulate the production of broad and effective immune responses, inducing sustained immune control of HIV-1 in the absence of therapy. A number of studies have explored the therapeutic potential of vaccination in both simian immunodeficiency virus (SIV) models (De Rose et al., 2008; Fuller et al., 2012; Fuller et al., 2006; Hel et al., 2002; Hel et al., 2000; Lu et al., 2003) and in human trials (Barouch et al., 2013; Garcia et al., 2013; Lévy et al., 2014; Lu et al., 2004) with vaccine agents including DNA based vaccines expressing antigen, viral vectors expressing antigen, passive transfer immunotherapy, dendritic cells (DC) primed for HIV-1 antigen presentation or combinations of these (Mylvaganam, Silvestri, and Amara, 2015). Generally, these studies have demonstrated that therapeutic vaccination can achieve reduced viral loads, increased time to viral rebound, reduction in size of the LR and in inducing stronger and more sustained immune response against HIV-1 (Mylvaganam et al., 2015).

Alternatively, strategies which aim to completely eradicate the HIV-1 LR are popular in current research and clinical trials (Kim, Anderson, and Lewin, 2018). These “shock and kill” approaches utilise latency reversing agents (LRA) to induce activation of latently infected cells in the presence of ART, rendering those cells susceptible to cytolysis or immune clearance whilst limiting the chance of subsequent rounds of infection (Archin et al., 2017; Archin et al., 2012; Elliott et al., 2015; Margolis et al., 2016). Adding to this, recent approaches have explored the potential of a “lock in and apoptosis” strategy that when combined with the LRAs, utilises a novel compound to antagonise the viral gag protein and prevent virus budding whilst still inducing virus apoptosis (Tateishi et al., 2017).

Research focused on the reduction or elimination of the LR must utilise robust assays that can reliably and reproducibly measure the effect that the treatment or vaccine strategy has on the size of the LR. The quantification of HIV-1 DNA from peripheral blood mononuclear cells (PBMC) of patients via polymerase chain reaction (PCR) provides a useful tool to monitor the size of the viral reservoir, and a number of assays have been developed targeting different regions of the HIV-1 genome including gag, *pol* and the long terminal repeat (LTR) (Beloukas et al., 2009; Kabamba-Mukadi et al., 2005; Rouzioux and Avettand-Fenoël, 2018; van der Sluis et al., 2013; Vandergeeten et al., 2014; Yun, Fredriksson, and Sonnerborg, 2002). The strength of these assays is the rapid turn-around from sample collection to DNA quantification and the possibility to identify different HIV-1 DNA forms, such as integrated DNA, unintegrated linear DNA forms and 2-LTR circular DNA (De Spiegelaere et al., 2014; van der Sluis et al., 2013; Vandergeeten et al., 2014). These different HIV-1 DNA forms have been used as markers of HIV-1 persistence in a number of different studies, reviewed here (Ruggiero et al., 2017). 2-LTR circular DNA is a product of abortive integration, and while some studies have suggested they are stable in CD4+ cells (Pace, Graf, and O'Doherty, 2013), they are considered markers

of recent infection and ongoing replication notwithstanding therapy (Chun et al., 1997b; Koelsch et al., 2008; Zhu et al., 2011). Only assays targeting the viral LTR allow for the discrimination of different HIV-1 forms in addition to the fact that the LTR contains some of the most conserved regions of the viral genome (van der Sluis et al., 2013). We have comprehensively analysed two HIV-1 DNA quantification assays, herein referred to as Vandergeetan, (CV) (Vandergeeten et al., 2014) and van der Sluis (RvS) (van der Sluis et al., 2013), both of which target highly conserved regions in the long terminal repeat (LTR) region of the virus genome (van der Sluis et al., 2013; Vandergeeten et al., 2014) (Fig. 1). Both assays utilise a PCR pre-amplification step with primers designed to only amplify DNA which has been fully reverse transcribed, excluding the short abortive transcripts. Additionally, both assays are able to distinguish between total HIV-1 DNA and 2-LTR circular DNA with the use of alternative primer sets in the pre-amplification step. The CV assay is also able to distinguish integrated HIV-1 DNA via the use of primers targeting human *Alu* sequences, randomly dispersed in the human genome (Ruggiero et al., 2017; Vandergeeten et al., 2014). A prominent HIV-1 LTR based DNA assay, herein referred to as Rouzioux (CRx), was excluded from this comparison because this assay does not distinguish between different DNA types (Rouzioux, Melard, and Avettand-Fenoel, 2014). Furthermore, we have evaluated several calibration cell-lines, aiming to establish a stable and reproducible source of HIV-1 DNA for use as a standard curve. Additionally, we have analysed the primer sequences and used this information to establish an assay with broader specificity and increased sensitivity.

2. Materials and Methods

2.1. Cell Lines and Calibration Standards. HIV-1 quantification standards were produced from cell lines including 8E5 (CFAR 95), ACH-2 (CFAR 349) and J-Lat 10.6 (CFAR 9849), obtained from the NIH AIDS reagent program. Additionally, we utilised SupT1-14 a previously characterised cell lined containing 14 HIV-1 copies per cell in comparing the assays (van der Sluis et al., 2013). Standards for the quantification of cell input were produced from dilutions of DNA derived from HEK293T cells (ATCC CRL-3216). ACH-2, 8E5 and J-Lat 10.6 were maintained in RPMI-1640 medium (Fisher, 11875093) supplemented with 10% heat inactivated FBS (Sigma, non-US origin, F7524) and 1% pen-strep (Fisher, 15140122) at 37 °C with 5% CO_2. HEK293T cells were maintained under the same conditions with advanced DMEM (Fisher, 12491015) used for culturing. Cells were passaged to a maximum of 10 cycles prior to DNA extraction using QIAamp DNA Blood Mini Kit, according to the manufacturer's instructions (Qiagen, 51104). DNA concentration and purity was assessed by Nanodrop analysis (Thermo Scientific, ND-200). The total number of cells and HIV-1 copy numbers were quantified using the CD3 and LTR quantification assays, respectively, and as previously described (van der Sluis et al., 2013; Vandergeeten et al., 2014). Standards were produced via a dilution series over a range of 5 logs. HIV-1 DNA standards were spiked with uninfected human genomic DNA to equalise DNA input in lower copy numbers.

2.2. Study Population Clinical Sample Preparation. The present study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois and all subjects provided written informed consent. CD4+ cells were isolated from PBMCs by negative selection using paramagnetic beads (StemCell Technologies) according to supplier's protocol. Purified CD4+ cells were digested via incubation with 0.1 mg/ml recombinant proteinase K (Roche, RPROTK-RO) in 10 nM Tris (pH 8.3) for 2 h at 56 °C. Lysate was centrifuged and

supernatant recovered and used as input in the HIV-1 quantification assays. Proteinase K lysates were stored at -80 °C until use.

2.3. HIV-1 DNA Quantification Assays. Total HIV-1 DNA was quantified using both CV (Vandergeeten et al., 2014) and RvS (van der Sluis et al., 2013) LTR based HIV-1 DNA as well as CD3 cell quantification assays. Primers and probes used in HIV-1 and CD3 DNA quantification are as described previously (van der Sluis et al., 2013; Vandergeeten et al., 2014). Additionally, a commercial cell quantification kit targeting the β-actin gene was used (ABI Applied Biosystems TaqMan β-actin Detection Reagent, 401846). Pre-amplifications were performed in 25 µl reactions using Taq polymerase (Invitrogen, 10342020), as previously described (van der Sluis et al., 2013; Vandergeeten et al., 2014). Quantifications were performed in 20 µl reactions using Supermix-UDG (Invitrogen, 11730025) with the Qiagen Rotor Gene RotorQ, as described previously (van der Sluis et al., 2013; Vandergeeten et al., 2014). β-actin quantifications were performed according to the manufacturer's instructions. Reagent mixes for the quantification and pre-amplification PCR steps were adapted to the volumes used in this study, though the final concentrations remained the same as previously described (van der Sluis et al., 2013; Vandergeeten et al., 2014).

2.4. Sanger DNA Sequencing. To sequence the primer and probe binding regions of both assays primers were designed in house to amplify the LTR region of patient samples (Table 1). Nested PCR was performed under the following conditions: 2 min (95 °C) followed by 35 cycles of 30 s (95 °C), 30 s (55 °C) and 1 min (72 ◦C) with a final elongation of 10 min (75 °C). The product of PCR 1 was diluted 1/10 in molecular grade water and this dilution was

192 subsequently used as input for PCR 2. Amplification was analysed using gel electrophoresis
193 and further purified using a Qiagen PCR Purification Kit (28104) prior to sequencing (GATC
194 Biotech and Source Bioscience). Patient sequences were then aligned to primer and probe
195 sequences using BioEdit software to identify mismatches. Following this, new primers were
196 selected to exactly match the patient sample LTR region and used to quantify the total HIV-1
197 DNA using both assays, as described above (Table 1).
198
199 **3. Results**
200 **3.1. Validation of assay quantification standards.** Our aim was to examine the performance
201 of the two assays CV and RvS and using the vast amount of sequence information available to
202 date develop a new assay that will perform most optimally with the highest specificity and
203 sensitivity. The incentive for this consideration was that both HIV-1 DNA quantification assays
204 target the LTR of the HIV-1 genome, well established as the most conserved region of the
205 genome, and furthermore both utilise a pre-amplification step allowing for the separate
206 quantification of different viral life-cycle stages. In order to do so we initially aimed to define
207 the cell quantification standard using a human genomic DNA input based on 293T cells. We
208 quantified the cell number using two methods; a commercial assay with primers targeting the
209 human β-actin gene and a previously described assay targeting the human CD3 gene
210 (Vandergeeten et al., 2014). We tested a 5 log standard range (10^5 to 10^1 cell equivalents)
211 using both assays and found that they were within the optimum range of amplification
212 efficiency (90-110%) and that there was no significant difference between either over 3 runs
213 (P=0.8538) (Fig. 2A and 2B). Based on this result we selected the CD3 quantification assay
214 because it includes a pre-amplification step consistent with the HIV-1 DNA assays.

Further, we ran the two HIV-1 quantifications assays, RvS (van der Sluis et al., 2013) and CV (Vandergeeten et al., 2014), using 5-log serial dilutions (10^5 to 10^1 HIV-1 copies per input) of the J-Lat 10.6, 8E5, SupT-14 and ACH2 cell lines. We found no significant difference between qPCR efficiency of both assays over 6 runs (P=0.0552). We next compared the HIV-1 DNA content in these cell lines, aiming to determine the most appropriate cell line for use as a quantification standard.

3.2 Evaluation of calibration cell lines. We evaluated HIV-1 integration model cell lines including ACH2, 8E5 and J-Lat as well as in 'in house' cell line, SupT-14, for their use as calibration standards. Cell lines were grown to 10 passages and the total HIV-1 per 10^6 cells was quantified following DNA extraction. Consistent with recent publications, we showed that HIV-1 copies per cell decreased in 8E5 cells from 1 to 0.2 copies (Fig. 3). Additionally, HIV-1 copies in ACH2 cells were found to increase from 1 to 4 copies per cell. On the contrary, HIV-1 DNA content was stable in both J-Lat 10.6 and SupT1-14, which contain 1 copy per cell consistent with recent studies (Sunshine et al., 2016) and 14 copies per cell as demonstrated previously (van der Sluis et al., 2013), respectively (Fig. 3). Based on these findings we used the J-Lat 10.6 to quantify patient samples in this study.

3.3 HIV-1 quantification in patient samples. We then compared the two HIV-1 DNA quantification assays using patient samples. Overall, a strong correlation was found between the results produced with the RvS and CV assays (r=0.9759, P<0.0001) (Fig. 4C). Nonetheless, the mean quantification of patient samples was significantly higher when using the RvS (3.385 Log10 HIV-1 copies/106 cells) HIV-1 assay compared to the CV assay (3.203 Log10 HIV-1 copies/106) (P=0.0021) suggesting a slight advantage of RvS over CV when testing patient

material (Fig. 4B). A possible explanation would that the CV amplified product is longer than the RvS thus affecting the amplification efficiency. Furthermore, the implementation of software (http://unafold.rna.albany.edu) revealing folded structures indicated that more complex folded structures of the CV amplicon could also account for a lower amplification efficiency. (Fig. 5) Of note, in 4/38 (10.52 %) of patients we observed a significant differences in quantification between the two assays (114: P=0.00101, 72: P>0.0001, 23: P>0.0001, 111: P=0.0003) (Fig. 4A).

We next aimed to test the performance of the two assays when HIV-1 copy input was diluted down to 10 copies. We found that in low copies, correlation was skewed towards the CV assay (r=0.8220, P<0.0001) and that in 9/25 (36%) of samples quantification was significantly different between the assays (Fig. 4D and 4F). However, there was no significant difference between the mean quantification of low copy patient samples (P=0.1456) (Fig. 4E).

3.4 HIV-1 quantification using patient tailored primers. We showed that both assays perform comparably; however, there is some discrepancy in quantification observed with some patient samples. We aimed to elucidate the cause of this discrepancy by sequencing the LTR of patient samples. Two forward and reverse primers were selected for nested LTR amplification based on identity with sequences of the Los Alamos database (Table 1). The LTR of patient samples was subsequently sequenced with the Sanger platform. Patient sequences were analysed using BioEdit and sequences were manually aligned to primer and probes used in both assays (data not shown). Based on this alignment, we selected primers tailored to patient samples (Table 1). Patient samples were quantified simultaneously with the universal and the patient tailored primers. The HIV-1 DNA copy numbers were significantly increased (131.9% and 141.6% average increase for RvS and CV assays, respectively) when matching

primers were used (Fig. 6A and 6B) with the corresponding patient samples demonstrating that sequence diversity can occasionally impair the accuracy of the assay.

We subsequently interrogated the sequence information available 'to date' at the Los Alamos HIV-1 database. Our in silico analysis revealed that the oligonucleotide with the higher propensity for mismatches was the RvS forward pre-amplification primer, at the 5' end (Fig. 7E), when compared to the other assay primers (Fig. 7A-D). We therefore redesigned this primer in two different versions (RvS-PreF-A and RvS-PreF-B, Table 1) (Fig. 7F), to compensate the sequence diversity and circumvent 5' end mismatches that would be the most deleterious. These two primer versions were used at equal ratio for the pre-amplification step. Our results indicate that the new primers yield a significantly higher quantification than the existing primer, and this represents and improvement on the assay (P=0.0116) (Fig. 8). Though this difference is small, our analysis suggests this primer combination will reduce the risk of mismatching in the 5' end of the primer and increase the overall coverage and accuracy of the assay. As it stands the *in silico* analysis showed that the overall primer diversity ranged between 0.04% and 0.07% as estimated using the neighbour-joining method and the Kimura-2-parameter model (data not shown) suggesting that the RvS primer combination will rarely underestimate the total DNA load.

4. Discussion

Clinical trials assessing the efficacy of therapeutic vaccination or HIV-1 eradication strategies must utilise robust and reproducible HIV-1 DNA quantification assays. The lack of a standard quantification assay to measure total HIV-1 DNA has led to the development of a

number of ‘in-house’ assays targeting different genomic regions for quantification, but this variation may render the results of different clinical trials incomparable. We selected two HIV-1 quantification assays, CV (Vandergeeten et al., 2014) and RvS (van der Sluis et al., 2013), for comprehensive evaluation to determine if results obtained were comparable and the assays could therefore be used interchangeably. These assays were selected for the ability to distinguish different HIV-1 DNA forms, including 2-LTR circular DNA, which can serve as a marker of recent infection and therefore be used to determine the success of treatment. The differential quantification of different DNA markers is facilitated by the use of a pre-amplification step and primers targeting the conserved regions of the LTR of the viral genome.

Recent data has suggested that 8E5, a commonly used latency model containing one copy of HIV-1 per cell, is unstable and rapidly loses HIV-1 copies during passaging (Busby et al., 2017; Wilburn et al., 2016). Further, a study has shown eveidence of ongoing replication within ACH2 cells during passaging, resulting in an increase in HIV-1 copies per cell (Sunshine et al., 2016). This study has proposed the use of J-Lat cells as quantification standards as these contain a non-replication competent copy of HIV-1 that remains stable after a number of passages (Sunshine et al., 2016). Consistent with these findings, we have compared a number of well characterised calibration cell lines and discovered that 8E5 and ACH2 cells are unsuitable for use due to the change in HIV-1 DNA copies during passaging (Busby et al., 2017; Wilburn et al., 2016). Further, we have demonstrated that J-Lat cells contain ~1 copy per cell and would therefore be the most suitable for used in DNA quantification studies. The universal use of only one cell line as a calibration standard would reduce variability of different HIV-1 DNA assays and across different labs, rendering data obtained from studies and clinical trials more comparable. Further, we demonstrate that both LTR based assays amplify well-characterised HIV-1 calibration cell lines with equal efficiency, removing the

311 potential of bias in quantification of patient samples arising from a bias in the amplification
312 of the standard curve.

313 Our data indicate that both assays perform comparably when quantifying total HIV-1
314 DNA in patient samples as well as cell lines and that these quantifications correlate strongly.
315 Despite this, we have shown that the RvS assay quantifies the patient set as a whole, 0.2
316 Log10 HIV-1 copies higher than the CV assay, suggesting that the quantification of patient
317 samples is more efficient when using this assay. When these samples were diluted to ~10
318 copies per input the strength of the correlation of the assays was lost. This is due to inherently
319 higher variation in the quantification of low copy samples, owing to the stochastic distribution
320 of template within the sample. However, the assay was improved when primers were
321 redesigned using sequences derived from a recent HIV-1 database.

322 The RvS assay utilises primers strategically placed to bind DNA only present following
323 first and second strand transfer of the reverse transcription process, ensuring that only fully
324 reverse transcribed DNA is amplified and therefore increasing the specificity and accuracy of
325 the assay (Figure 1) (van der Sluis et al., 2013). Based on this property we decided to improve
326 the assay using an analysis of sequences from the HIV-1 database. The high degree of HIV-1
327 sequence heterogeneity means that sequence variation will be encountered even within the
328 most conserved regions of the genome. Our analysis showed that the forward pre-
329 amplification primer was most divergent from published sequences and we therefore
330 redesigned this primer and suggest that two primers (Table 1) should be used to improve the
331 accuracy and sensitivity of this assay.

332 HIV-1 DNA quantification is an essential tool for monitoring HIV-1 vaccine and therapy
333 trials due to its low cost, fast turnaround time and high throughput capacity. Notwithstanding
334 its advantages, DNA based assays cannot distinguish between replication competent and

replication defective pro-virus, and will therefore overestimate the size of the replication competent LR (Rouzioux and Avettand-Fenoël, 2018; Ruggiero et al., 2017). Despite this, recent studies have suggested defective pro-virus contributes to HIV-1 pathogenesis, and so measuring the size of all pro-virus present in a sample is useful marker of vaccine or treatment success and projection for disease progression (Rouzioux and Avettand-Fenoël, 2018; Ruggiero et al., 2017). In any case these described assays are a cheaper, faster and more practical alternative to the cell based viral outgrowth assay (VOA) which is able to specifically quantify only replication competent pro-virus by measuring virus production in PBMCs following activation (Rouzioux and Avettand-Fenoël, 2018). Here we demonstrate that two HIV-1 quantification assays perform comparably and can therefore be used interchangeably in clinical settings. Furthermore, we have improved the RvS assay through increasing the coverage of the diverse HIV-1 populations that can be detected with the assay.

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FIGURE LEGENDS

Fig. 1. HIV-1 LTR region: Locations of primers and probes for the CV, RvS and CRx assays. The numbers indicate the position on the HXB2 genome. For CV assay forward quantification primer anneals to Lambda T heel sequence on the forward pre-amplification primer.

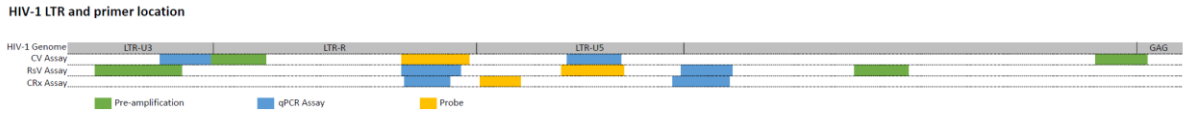


Fig. 1. Comparison of standards and assay performance: A) 5 log serial dilution of human genomic DNA quantified using CD3 and β-actin qPCR. B) Average amplification efficiency of CD3 and β-actin assays (n=3). C) 5 log serial dilution of J-Lat clone 10.6 cells using CV and RvS. D) Average amplification efficiency of CV and RvS assays.

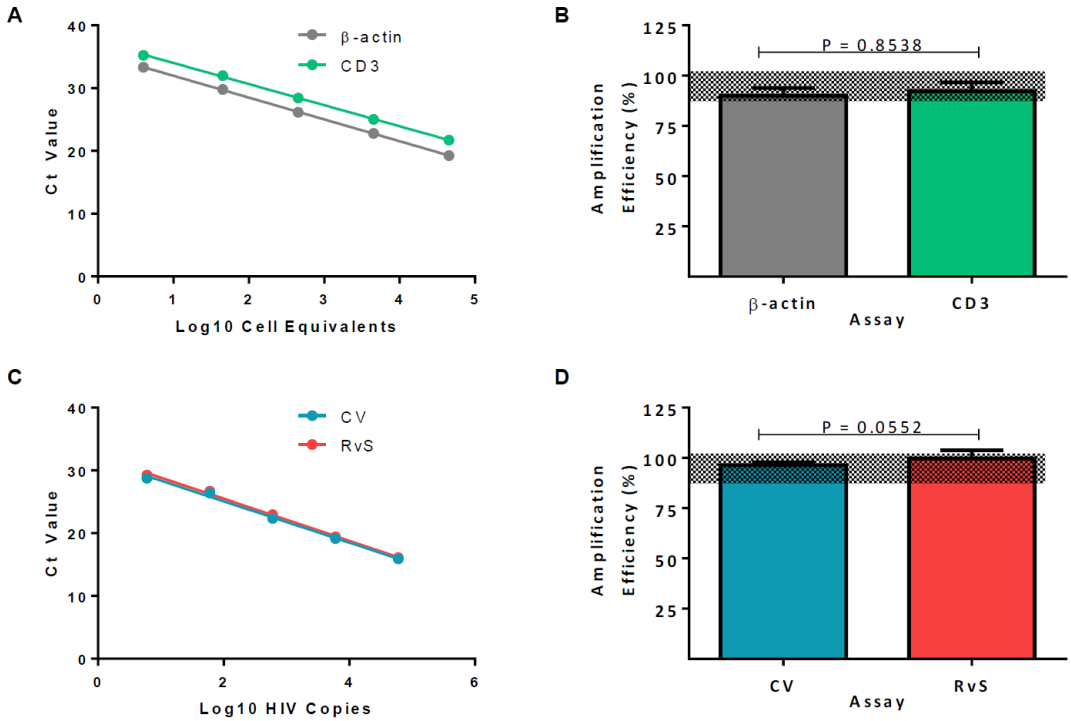


Fig. 3. Quantification using different cell lines: Quantification of three cell lines containing 1 HIV-1 copy per cell (8E5, J-Lat) and 14 HIV-1 copies per cell (SupT1-14). A five log dilution series for each cell line was performed and used as input for the assay. Quantification of each dilution was pooled and standardised to determine the average HIV-1 copies per cell.

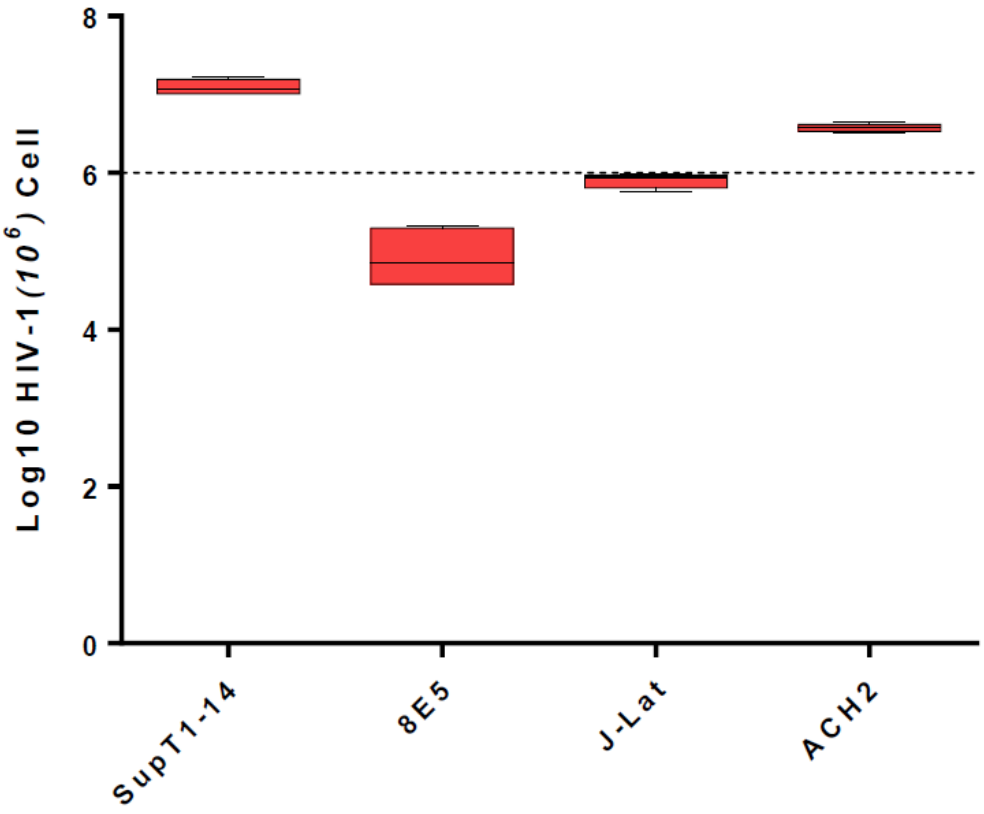
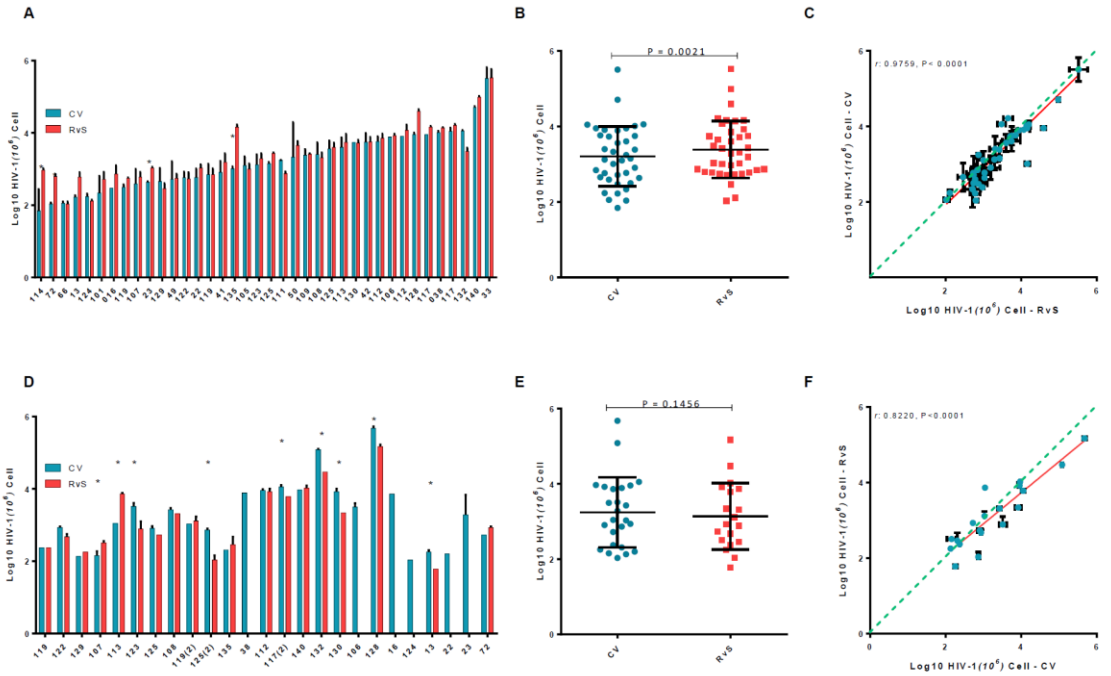


Fig. 4. Quantification of patient samples: A) Pellets of PBMCs or CD4+ cells extracted using proteinase K digestion or Qiagen DNA extraction. Total HIV-1 was quantified using RvS and CV assays and cells were quantified using the CD3 assay. Statistical significance determined using the multiple t-test, Holm-Sidak method, with alpha = 5.000%. B, E) Dot plot showing differences in mean quantification for undiluted and low copy quantification. Significance determined by paired t-test. D) Samples were diluted to 10 copies per reaction and quantified using both assays. Statistical significance determined using the multiple T test, Holm-Sidak method, with alpha = 5.000%. C, F) Correlation of all samples and correlation of diluted, low copy samples, respectively. Solid red line represents linear regression and green dashed line represents perfect correlation.

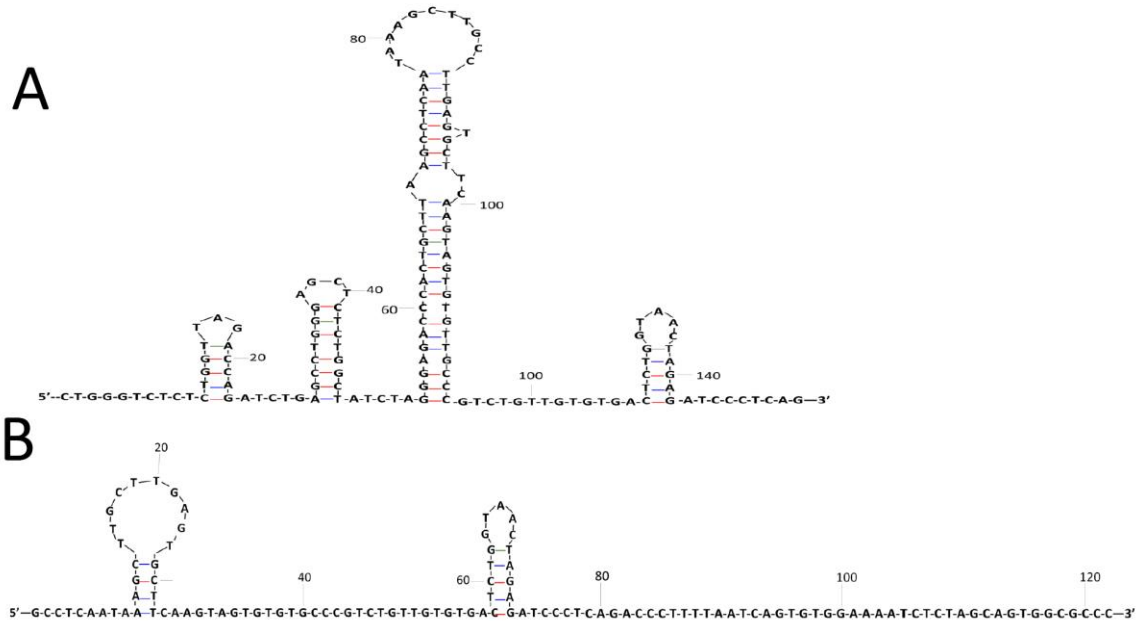


531

532 **Fig. 5.** The probable secondary structure of single stranded HIV-1 DNA produced using the

533 The mfold Web Server (http://unafold.rna.albany.edu): A) Depicts the 152 nt CV amplicon

534 (HxB2: 522→643) and B) Depicts the 122 nt RvS amplicon (HxB2: 452→603).



535

536 **Fig. 6.** Quantification with sequence matched primers: Primers designed to match sequences

537 were compared with assay primers: A) Comparison of CV primers to sequence matched

primers. B) Comparison of RvS primers to sequence matched primers. Statistical significance determined using the multiple t-test, Holm-Sidak method, with alpha = 5.000%.

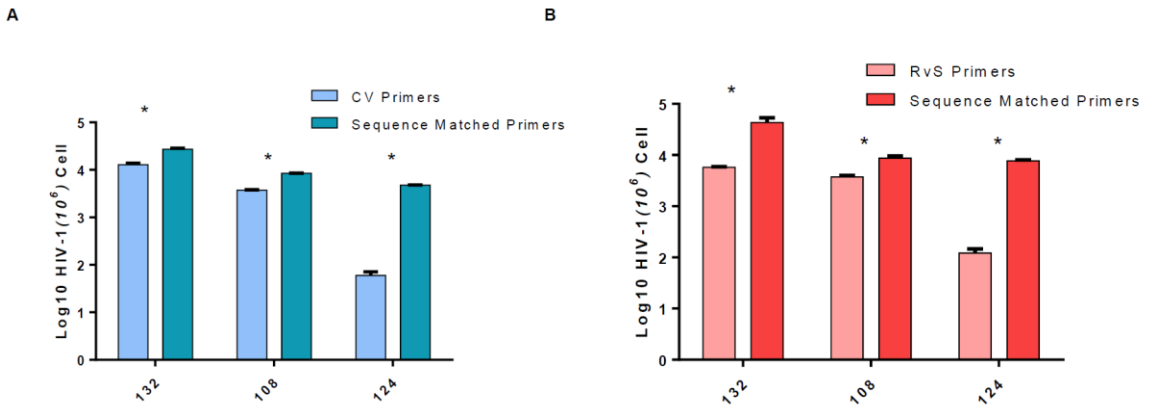
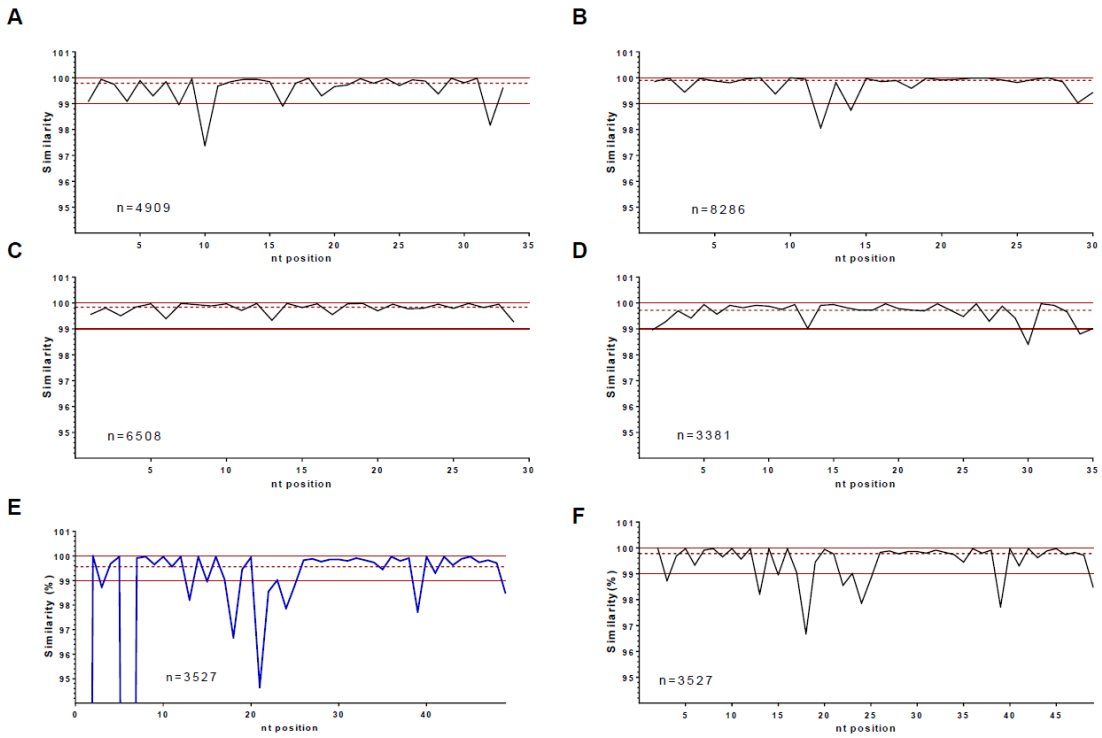


Fig. 7. Analysis of primer and probe sequence identity to published HIV-1 sequences: A) RvS probe sequence B) RvS pre-amplification reverse primer C) RvS qPCR reverse primer D) Region targeted by both VC Probe and RvS qPCR forward primer E) RvS pre-amplification forward primer F) Modified assay primer encompassing both primer A and B, where one has a nucleotide removed (Table 1) n = the number of sequences analysed per oligonucleotide.

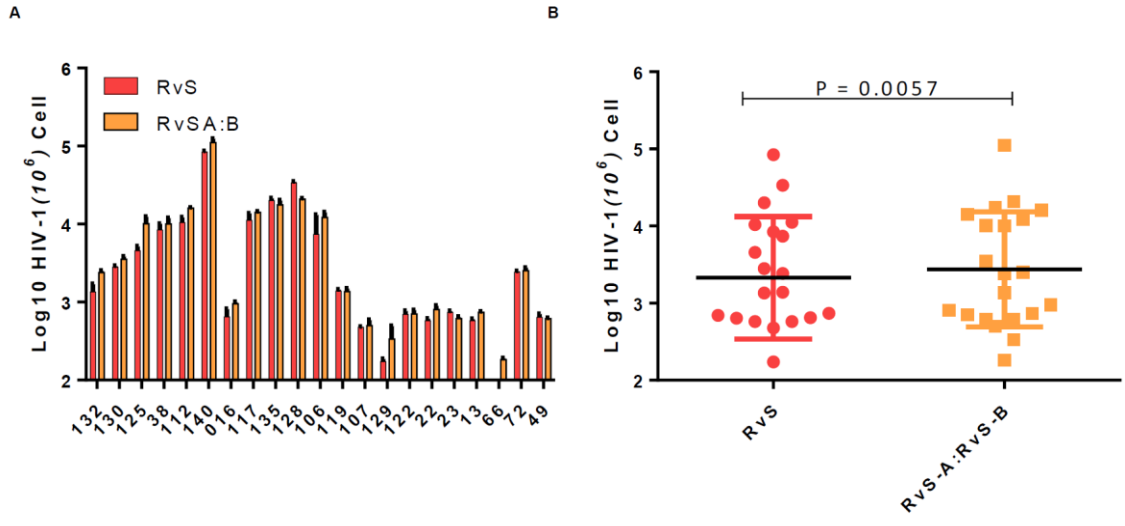


546

547 **Fig. 8.** Comparison of new compared to existing primers to quantify patient samples. Samples

548 were quantified with an equal mix of primer A and B (Table 1) to increase coverage of the

549 genome. Significance determined by paired t-test.



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551

552

TABLE 1 Primers used for the amplification of patient sample LTRs and primers matched to patient sequences. For tailored and redesigned primers nucleotide positions that vary from the universal assay primer are underlined.

Name	***Stage***	***Function***	***Sequence***	***Position on HXB2***
Seqout-F	Sequencing PCR 1	Forward	CACACACAAGGCTACTTCCCTGATTAGCAGAACT	57-90
Seqout-R	Sequencing PCR1	Reverse	CTTAATACTGACGCTCTCGCACCCATCTCTCT	815-784
Seqin-F	Sequencing PCR2	Forward	GGGACTTTCCGCTGGGGACTTTCC	350-373
Seqin-R	Sequencing *PCR2*	*Reverse*	*TCTCTCTCCTTCTAGCCTCCGCTAGTCA*	790-763
RvS-preF_132	*Pre-amplification*	*Forward*	*CAACCTTCAGAAGCTGCATAWAAGCAGCYGCT*	409-440
RvS -preR_132	*Pre-amplification*	*Reverse*	*AGCAAGCCGAGTCCTCGCGTC*	688-707
RvS -preF_108	*Pre-amplification*	*Forward*	*GAGCCCGTGGATGCTGCATAWAAGCAGCYGCT*	409-440
RvS -preR_108	*Pre-amplification*	*Reverse*	*AGCACAGCCGAGTCCTGCGTC*	688-707
RvS –qF_ 124	*qPCR*	*Forward*	*GGGCGCCACTGCTAGAGAA*	625-643
CV-preF_124	*Pre-amplification*	*Forward*	*ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGCTGGAC*	452-471
CV -preR_124	*Pre-amplification*	*Reverse*	*CCATCTCTCTCCCTTCTAGC*	775-793
CV -preF_132	*Pre-amplification*	*Forward*	*ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGCTAGAC*	452-471
CV –preF_108	*Pre-amplification*	*Forward*	*ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGTCAGA*	452-471
RvS-preF-A	*Pre-amplification*	*Forward*	*ARCCCTCAGAHGCTGCATAWAAGCAGCYGCT*	410-440
RvS-preF-B	*Pre-amplification*	*Forward*	*ARCCCTCAGAHGCTGCATAWAAGCAGCYGC*	410-439